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Innovative optofluidics and microscopy-based rapid analysis of pathogens

HAO WANG,[1] PRIYANKA SHIVESHWARKAR,[1] ROBERT BRZOZOWSKI,[2] ARSENY ZHDANOV,[1] SHULIN SHI,[1] PRAHATHEES ESWARA,[2] AND ANNA PYAYT[1,*]

[1]Department of Chemical and Biomedical Engineering, University of South Florida, 4202 E. Fowler Ave, ENB118, Tampa, FL 33620, USA
[2]Department of Cell Biology, Microbiology and Molecular Biology, University of South Florida, 4202 E, Fowler Ave. ISA 2015, Tampa, FL 33620, USA
*pyayt@usf.edu

Abstract: The timely knowledge and prescription of the most suitable antibiotic to treat bacterial infections is critical for the recovery of patients battling life-threatening bacterial infections. Unfortunately, current standard-of-care approaches relies on the empiric prescription of an antibiotic, as determination of the most effective antibiotic requires multiple time-consuming steps. These steps often include culturing of the bacterium responsible for infection and subsequent antibiotic susceptibility testing. Here we introduce an optofluidic technology that allows us to capture bacterial cells efficiently and rapidly from different biological samples and use the captured cells for rapid antibiotic selection thereby bypassing the need to culture the bacterium.

1. Introduction

Sepsis is a potentially lethal condition usually caused by enhanced immune response to an infection. It currently affects more than 1 million Americans per year with 15-30% mortality rate [1]. The number of sepsis cases per year has been on the rise in the United States, and rapid rise of antibiotic resistance poses a significant problem in how we treat these cases [2]. Bacteria, such as *Escherichia coli*, *Streptococcus pneumoniae*, and *Staphylococcus aureus*, are the most commonly encountered microorganisms in sepsis patients [3]. Antibiotics are prescribed to treat infections caused by bacteria including bacterial sepsis [4]. Generally, 2 days are required for the positive identification of the bacterial species causing infection and for the testing of candidate antibiotics to determine the most effective treatment option. Unfortunately, time is often limited, and as a result, antibiotics are prescribed empirically without proper testing. Consequently, treatments can often be inefficient, and the unneeded exposure of bacteria to antibiotics paves the way for rise in antimicrobial resistance. Additionally, reports show that many patients with sepsis exhibit negative blood culture results even after several days [5], indicating that it is possible to not detect some microorganisms using conventional approaches.

Similar time-related constraints are problematic in the testing of other biological fluids. For example, the laboratory examination of urine specimens accounts for a large part of the workload in hospital laboratories. Unfortunately, traditional approaches for processing urine cultures, and subsequent antimicrobial testing, requires 2 to 3 days [6]. This significantly delays treatment and negatively affects the outcome of the patient, if the empiric antibiotic prescription was suboptimal. As a result, there is an urgent need for new technologies that allow for prompt detection of bacteria in biological samples, followed by rapid antibiotic selection, to decrease the time it takes to inform and guide the prescribing physician.

Currently there are several alternative approaches focused on speeding up the identification of optimal antibiotics for treating infections. For example, PCR-based assays, such as multiplex-PCR,

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can deliver results differentiating between bacteremia vs fungemia in approximately 6 hours [7]. However, this requires additional steps to precisely identify the disease-causing agent and to perform the antibiotic susceptibility testing [8]. Another approach is to use mass spectrometry combined with the database of the peptide mass fingerprints of known pathogens to identify microorganisms [9]. Peptide nucleic acid (PNA) fluorescent in situ hybridization (FISH) stains (AdvanDx) are commercially available for direct identification of selected pathogens from positive blood cultures [10]. Electrochemical sensor assays can be applied to rapid (30 min) genotypic identification of bacterial pathogens [10]. Additionally, the Verigene gram-positive blood culture test can be used for identification of 12 gram-positive bacteria and 3 genetic markers of antibiotic resistance directly from positive blood culture medium [10]. Flow cytometry can be also applied to bacterial identification [11]. Unfortunately, the main drawback is that these techniques require culturing bacteria, and only can be conducted after significant time delays [10]. Additional time delay is introduced because of the antibiotic susceptibility testing.

An alternative approach is to use signature molecules, such as pathogen-associated molecular patterns (PAMPs) that are specifically present in bacteria and fungi and can be used to quickly identify the nature of the disease-causing agent. In fact, a recent study using enzyme-linked lectin-sorbent assay (ELLecSA) has showed promising results using this technique in identifying pathogens [12]. Other approaches include single molecule scanning (SM-Scanning) [13,14], or electrochemical-based detection that depends on the presence of 16S rRNA of bacteria [15]. In line with the other methods that are being developed to address this major problem in pathogen analysis, we propose a new approach to analyze biological fluids using an optofluidic technology developed by our laboratory [16] for rapid concentration of bacteria followed by rapid antibiotic screening. There are several other methods focused on the use of microfluidics for isolation of bacteria from blood [17,18]. One of them was focused on isolation and concentration of bacteria from blood using microfluidic membraneless dialysis and dielectrophoresis. This approach is very different from proposed here since it is based on electrical properties of all components, and also requires change of the conductivity of the fluid to operate. Another interesting approach to bacterial isolation is based on elasto-inertial microfluidics. It achieves good isolation performance and works reliably with whole blood, but currently can only process 60 µl/hour, even though some parallelization designs were proposed [18].

2. Method

The first step required for antibiotic selection testing is isolation of bacteria from a biological sample. There are two challenges that we face while isolating bacteria from the samples. First, bacteria might be present in low concentrations and thus must be preconcentrated. Second, there are many other particles and cells present in those samples. This makes traditional techniques based on micro-scale filtering challenging, since it is much easier to isolate larger objects (e.g. circulating tumor cells or blood cells), than tiny bacteria present in low concentration. Because of that we are proposing to use optofluidic particle manipulation that allows us to selectively concentrate micro-scale objects of a desired size that we previously demonstrated on a variety of micro-particles and cells.

Figure 1 shows the schematics explaining the mechanism of optofluidic concentration of bacteria. One of the critical components of the system is a specially designed bi-metallic substrate. It consists of a glass slide with sputtered layers of chromium and gold optimized for efficient absorption of green light and local fluid heating. In the prior publication we conducted detailed simulations and experimental optimization of the substrate and demonstrated that it works most efficiently when we use glass slides with sputtered 5 nm of chromium and 200 nm of gold. The wavelength of the laser used in all manipulations 532. All fluorescence microscopy is conducted from the top side of the substrate, while laser heating is done from under the substrate. This

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way sample is not exposed to the laser light and quality of the fluorescence imaging is not compromised.

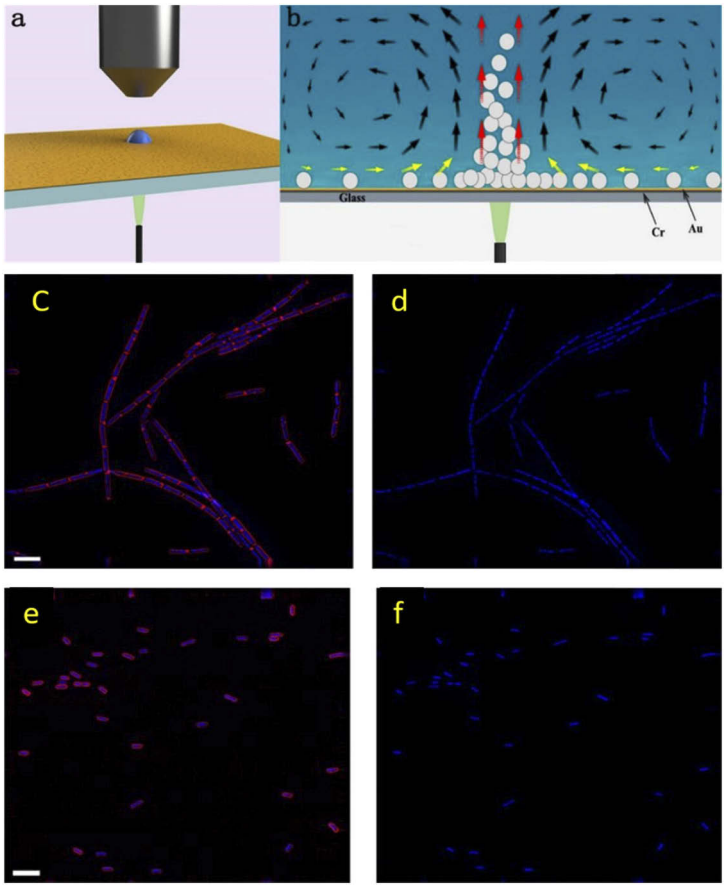

Fig. 1. Experimental setup and bacteria used for manipulation. a) A 3D model of the experimental set up for optofluidic manipulation of bacteria. The bi-metallic substrate is horizontally placed in between the microscope objective and an optical fiber with a metallic layer facing up. The lens and the optical fiber are aligned, so the spot on the substrate exposed to the light from the fiber is imaged using the objective. The liquid sample is dropped on the substrate, and then light is turned on. B) shows a zoomed-in cross-section of the droplet on the substrate after light is turned on. The symmetric vortex is formed, and bacteria shown as white circles are concentrated around the heated spot. Image c) and d) show *B. subtilis* wild type strain PY79. Image e) and f) show *E. coli* wild type strain K12. Both species have their cell membrane stained red with FM4-64, which can be observed in c) and e). Blue fluorescence indicates the location of DNA inside cells via DAPI stain. Scale bar is 5 µm.

Figure 1(a) shows a drop of fluid sample under a microscope objective on a bi-metal substrate. A cleaved tip of an optical fiber is placed under the substrate. Light is coupled into this fiber from a laser with a wavelength of 532 nm and an adjustable power. Light from the fiber tip locally illuminates the substrate, gets partially absorbed by the bi-metallic layer and locally heats the fluid. Warm fluid rises to the top, and this results in the generation of a toroidal convection current (Fig. 1(b)). The horizontal part of the current, shown with yellow arrows, can be used to trap micro-scale particles, while the vertical section, shown in red, can be used to levitate them. This setup can be used to capture, sort, and separate particles based on their size [16]. For

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example, for the fluids containing a mix of particles of two significantly different sizes, we can independently control each particle population. With a low laser power we can capture small particles without moving large ones. Alternatively, with higher laser power we can capture both large and small particles, but then remove and separate small ones by levitation, while keeping large ones on the surface of the substrate [16]. Therefore, particles can be controllably separated based on size. For our specific application we concentrate bacteria without moving other cells present in the sample and without phototoxicity or significant heating.

In these experiments we used two types of bacteria (gram-positive and gram-negative) with very different cell characteristics to demonstrate that our approach works equally well for different bacterial species (Fig. 1(c-f)). Wild type *Bacillus subtilis* (PY79), which is a gram-positive model organism, often forms chains of cells during exponential growth (Fig. 1(c) and (d)). Wild type *E. coli* (K12) is a gram-negative model organism (Fig. 1(e) and (f)). Bacterial cells were stained by 4',6-diamidino-2-phenylindole (DAPI) in order to visualize DNA. When bound to double-stranded DNA, DAPI has an absorption maximum at a wavelength of 358 nm (ultraviolet) and its emission maximum is at 461 nm (blue). Also, the cell membranes of the bacterial cells were stained with FM4-64 fluorescent dye (excitation/emission maxima 515/640 nm), emitting red fluorescence signal.

3. Results

3.1. Concentration of bacteria

We have previously demonstrated that we can concentrate particles and cells in water and buffer [16], however real biological samples are much more complex, and have a variety of particles and cells of different sizes. The goal of the following experiments was to demonstrate that size-dependent isolation of bacteria can be conducted in real biological samples. The experimental condition should be altered depending on the bacterial load in samples.

Figure 2 shows a diluted rat blood sample spiked with bacteria. Whole blood was diluted with standard phosphate buffer saline (PBS) 2000 fold, and then mixed with *B. subtilis* cells (CFU approximately equal to $2*10^7$ per ml) the volume ratio of 1:1. The final sample volume was 50 μl and the laser power was 30 mW. Image a) and d) are the 3D rendering made using Autodesk Inventor created for easier understanding of the difference between the experiments. Parts b, e show bright field microscopy images with dark dots representing individual red blood cells, while both figure c and f are fluorescence images taken in the same field of view, but showing DAPI-stained bacteria.

At first, both bacteria and red blood cells are evenly distributed on the substrate. After 2 min of optofluidic concentration, bacteria aggregated in one spot. Figure (b) shows cell distribution before optofluidic manipulation and e) after manipulation, while bacteria are difficult to see because of the small size and transparency. Figure (c) and (f) are fluorescence microscopy images taken under UV excitation. Since *B. subtilis* cells are stained by DAPI, the cells emit blue fluorescence with a peak at 461 nm. Image c) shows that bacteria are initially distributed evenly. After two minutes of optofluidic manipulation bacteria aggregate above the optical fiber (Fig. 2(f)). This way we can independently track populations of the RBCs and bacteria and demonstrate local concentration of bacteria in a blue spot (Figure (f)) with much darker area around the spot.

The left part of the Fig. 2 demonstrates that relatively high concentrations of bacteria can be manipulated using optofluidics, and the right part shows a similar experiment conducted at much lower concentration of bacteria. The bacterial sample was diluted 2000-fold (100X less concentrated than in the left images). The diluted bacteria sample was mixed with diluted whole blood at a volume ratio of 1:1. Since the initial number of bacterial cells is low (CFU reaches as low as $5*10^3$ per ml), initially there are no bacteria in the field of view of the microscope, and only

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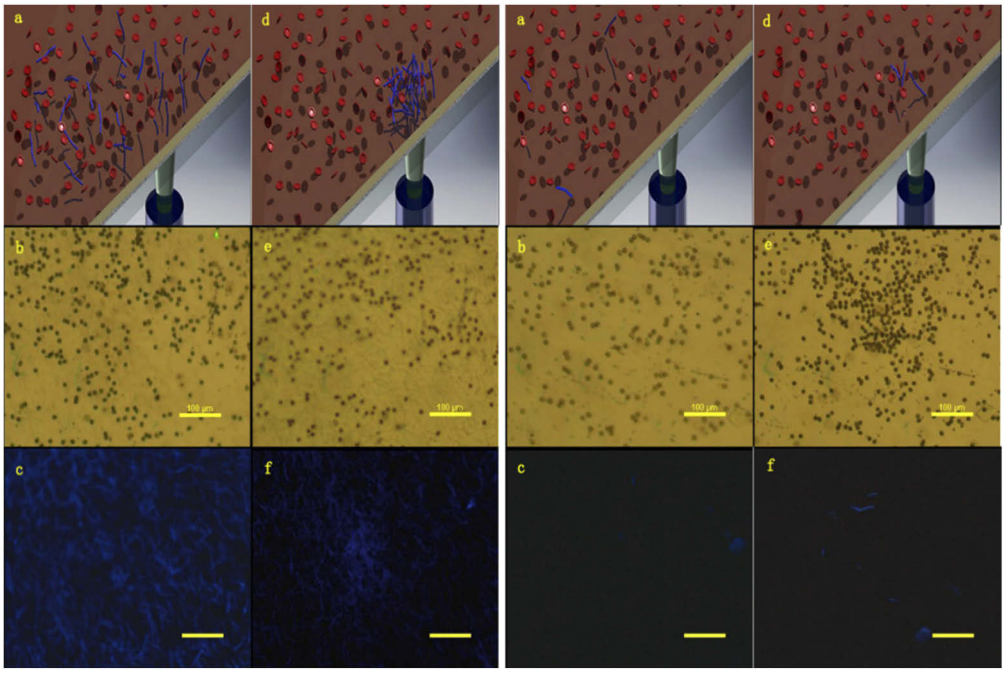

Fig. 2. There are two sets of images that demonstrate that both high (left set) and low (right set) concentrations of bacteria can be aggregated after 2 minutes of optofluidic manipulation. Images a) - c) show the original distribution of cells and d) - f) show cell distribution and bacteria aggregate after optofluidic manipulation. a) and d) are artistic renderings, b) and e) bright field images, and c) and f) fluorescence images. Laser power was 25 mW in both tests. Scale bar on each image is 100 µm.

after the optofluidic concentration we are able to see several bacterial cells. This demonstrates that optofluidic concentration of bacteria works for a wide range of concentrations of cells.

The next experiment demonstrates that the optofluidic bacterial concentration works equally well for bacteria with very different cell characteristics and for a different type of biological specimen. Here a sample was prepared by mixing fibroblast cell suspension with *E. coli* in the volume ratio of 100:1. At first, we cultured human lung fibroblast cell (IMR-90) in a petri-dish. Fibroblast cells, unlike blood cells, are anchorage-dependent cells which can only grow when attached to a substrate. To harvest the cells, a standard protocol for cell detachment was used. As soon as we obtained fibroblast cells suspension, it was immediately mixed with the bacterial suspension (CFU approximately equal to $1*10^9$ per ml). The sample volume was again 50 µl, and the laser power was 30 mW. Figure 3(a) is a bright field image after optofluidic concentration of bacteria, while Fig. 3(b) is a fluorescence image. The blue spot in the Fig. 3(b) is a cloud of concentrated bacteria. Interestingly, in Fig. 3(a) we observed a dark spot in the bright field image. This indicated that, at least for *E. coli* the concentrated bacteria can be observed in the bright field. Figure 3(c-e) shows that the cloud of bacteria accumulates over time. Initially it is absent (Fig. 3(c)), then it starts forming after one minute of optofluidic manipulation (Fig. 3(d)). Finally, many bacterial cells are captured after one more minute (Fig. 3(e)). Figure 3(b) shows that the area around the bacteria cloud is free of other cells, demonstrating that most of the bacteria from the sample is now concentrated in a single spot.

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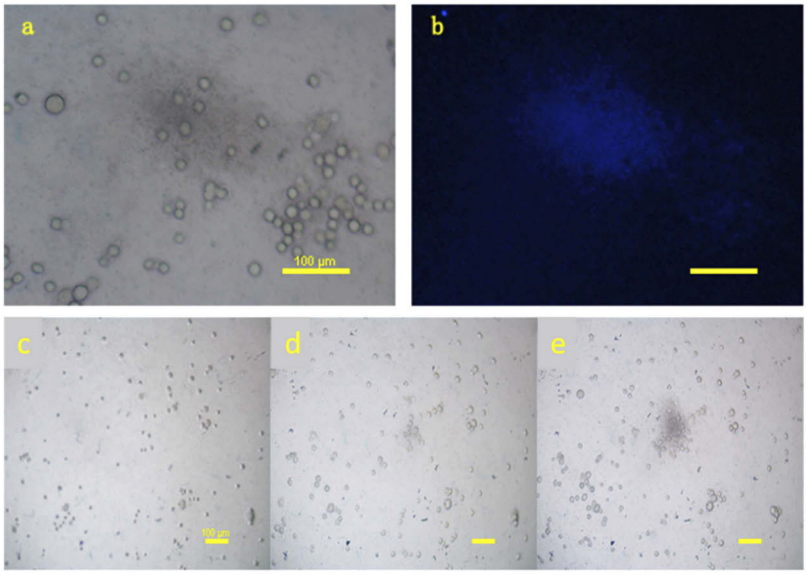

Fig. 3. Optofluidic concertation of *E. coli*. *E. coli* cells were stained with DAPI emitting blue fluorescence light under UV illumination. a) The round transparent objects are fibroblast cells, they do not move under optofluidic manipulation. b) The blue cloud is a fluorescence image of the bacteria concentrated in a single spot. c)-e) show gradual formation of the bacterial cloud. Scale bar is 100 µm.

3.2. Isolation of bacteria

Concentrated bacteria have to be isolated from the sample and used for antibiotic screening. It can be done by integrating the optofluidic manipulator on a microfluidic chip. Figure 4(a) shows the microfluidic setup used for capturing of the concentrated bacteria with a thin needle. The sample is injected into a microfluidic channel through the inlet, the bacteria are concentrated, and removed using a thin sharp needle which was prefabricated inside of the channel. The optofluidic bacteria concentration step isolation step is conducted in front of the needle tip. After that, the small amount of fluid with the concentrated bacteria can be pumped through the needle outside of the microfluidic channel for further antibiotic screening. Subsequently, the fluid sample with removed bacteria is pumped out through the second outlet, and "concentrate-isolate-pump" steps can be continuously repeated to process larger sample volume. In the future we plan to integrate isolation chip with the screening chip into one continuously operating microfluidic system.

In the experiment in Fig. 4(b-I), DAPI-stained *B. subtilis* cells were injected in a microfluidic channel, and the bacterial cloud was captured after 10 min of optofluidic concentration. It can be noticed as a flow of dark/blue material in a direction of the needle tip (Fig. 4(f,g)). The captured bacteria were released on a different substrate and were concentrated again (Fig. 4, h-i and Fig. 5). Fluorescence emitted by the bacteria can be used to determine transfer efficiency. We use the Photoshop Histogram tool to extract the integrated blue color intensity of the section inside yellow circle in both images. It was demonstrated that ~60% bacteria could be successfully captured, transported to a different substrate and re-concentrated.

3.3. Rapid antibiotic screening

In order to test our ability to perform rapid antibiotic screening for different types of bacteria, we imaged wild type and ampicillin-resistant (AmpR) *E. coli* (a gram-negative bacterium) and *S. aureus* (a gram-positive bacterium). The cells of these strains were treated with ampicillin, a

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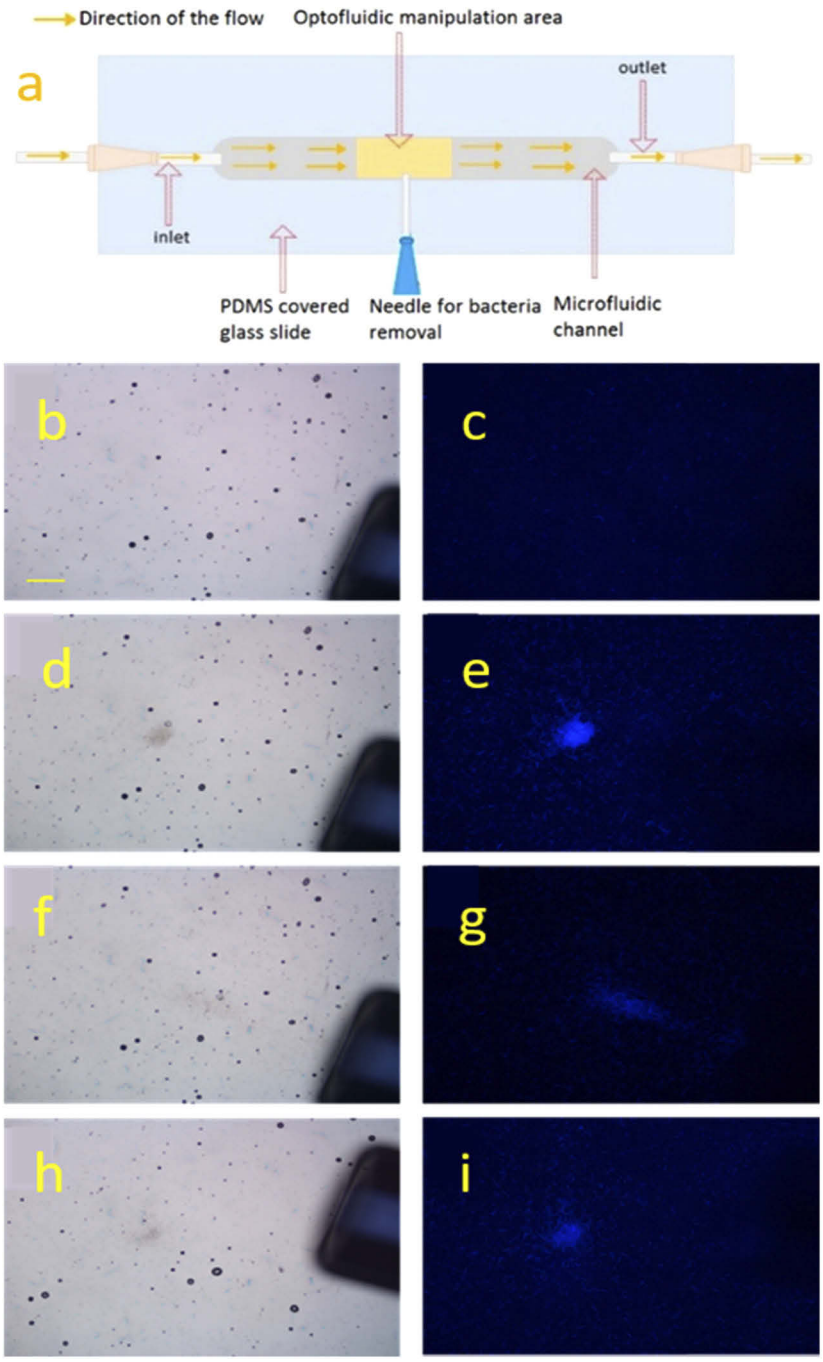

Fig. 4. Schematics of an experimental setup with a microfluidic channel for capturing of concentrated bacteria for further analysis followed by experimental results with DAPI-stained *B. subtilis*. The bright field and fluorescence images were obtained over the same field of view to demonstrate the position of the needle and the bacteria. The optofluidic concentration was conducted for 10 min using 25 mW laser power, and bacteria were captured in one bright spot. Then the bacteria cluster was removed through a syringe needle connected to a microfluidic pump through tube (flow rate was <10 µl/min). The bacteria cluster in 50 µl of solution was released onto a new chip for further analysis (Fig. 5). The scale bar is 100 µm.

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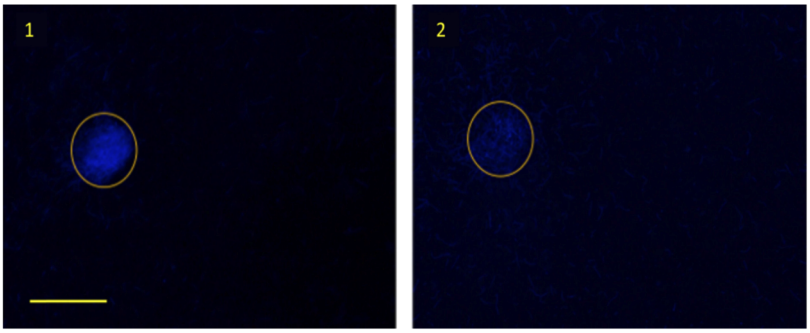

Fig. 5. Magnified view of the initial concentrated cluster of bacteria (1) and one obtained after re-concentration (2). Both images were taken under identical imaging conditions, and at least 60% of bacteria was successfully captured, transferred and re-concentrated. Laser power was 25 mW. The scale bar is 100 µm.

bactericidal antibiotic that inhibits peptidoglycan synthesis and causes cell lysis. As shown in Fig. 6, while the wild type cells were susceptible to ampicillin (as indicated by cell lysis), as expected, ampicillin-resistant *E. coli* and *S. aureus* cells did not show any lysis upon ampicillin treatment. The concentration of ampicillin (8 µg/ml) selected closely follows the clinically relevant minimum inhibitory concentration.

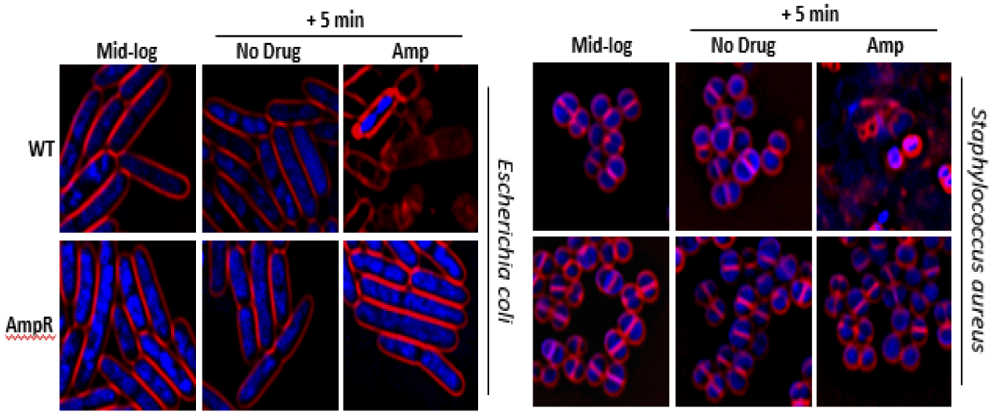

Fig. 6. Fluorescence microscopy was used to determine the susceptibility of wild type (WT) and ampicillin-resistant (ampR) *E. coli* cells as well as WT and ampR *S. aureus* cells to the treatment of ampicillin. Cells were grown to mid-logarithmic phase and an aliquot of sample was imaged. Next, cells were treated with 8 µg/ml ampicillin for a period of 5 min and subsequently imaged to determine susceptibility to the drug. Untreated cells grown for the same duration of time at the same temperature were used for comparison. Cells were stained with DAPI (DNA; blue) and FM4-64 (membrane; red) and imaged at mid-log phase growth (no drug), 5 min later (no drug), and 5 min later (drug). Ampicillin-sensitive strains lysed in the presence of the drug, whereas ampicillin-resistant cells were able to survive ampicillin treatment. This demonstrates that real-time testing of bacteria with antibiotics can be easily monitored under the microscope.

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4. Conclusion

We have demonstrated that different types of bacteria in various biological samples can be efficiently concentrated and isolated for further testing. The concentration approach works for diluted blood and cell growth media. Presence of cells does not prevent efficient concentration of bacteria. This approach works well for bacteria with very different cell structure, and for a wide range of concentrations from under $2.5*10^2$ cells in a 50 µl sample to 100X more concentrated. The concentrated bacteria can be isolated from the sample using a microfluidic chip and a thin needle, transferred to a different substrate and re-concentrated with at least 60% efficiency. The live bacterial sample can be analyzed using rapid antibiotic screening using microscopy to observe morphological changes produced by the administration of an antibiotic. Significant difference was observed after administration of the drug to the wild type and the drug-resistant strains. The testing is done optically on a small number of live bacteria and the susceptibility to different antibiotics can be visualized within minutes from the beginning of the assay. Once bacterial cells are isolated from the biological sample, the effectiveness of bactericidal antibiotics can be determined in approximately 1 hour. This is significantly less time than the standard 24-48-hour cultures that are generally required using a conventional approach. Overall, this technology can greatly speed up the identification of appropriate antibiotic in case of bacterial infections.

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Disclosures

The authors declare no conflicts of interest.

References

1. E. K. Stevenson, A. R. Rubenstein, G. T. Radin, R. S. Wiener, and A. J. Walkey, "Two decades of mortality trends among patients with severe sepsis: a comparative meta-analysis," Crit. Care Med. **42**(3), 625–631 (2014).
2. G. Kumar, N. Kumar, A. Taneja, T. Kaleekal, S. Tarima, E. McGinley, E. Jimenez, A. Mohan, R. A. Khan, J. Whittle, and E. Jacobs, "Nationwide trends of severe sepsis in the 21st century (2000–2007)," Chest **140**(5), 1223–1231 (2011).
3. Gizem Polat and Rustem Anil Ugan, "Sepsis and Septic Shock: Current Treatment Strategies and New Approaches," Eurasian J. Med. **49**, 53–58 2017.
4. S. Y. Liang and A Kumar,, "Empiric antimicrobial therapy in severe sepsis and septic shock: optimizing pathogen clearance," Curr. Infect. Dis. Rep., **17**(7), 36 (2015).
5. I. M. Sheldon, "Detection of Pathogens in Blood for Diagnosis of Sepsis and Beyond," EBioMedicine **9**, 13–14 (2016).
6. L. B. Reller, M. Weinstein, J. H. Jorgensen, and M. J. Ferraro, "Antimicrobial susceptibility testing: a review of general principles and contemporary practices," Clin. Infect. Dis. **49**(11), 1749–1755 (2009).
7. L. E. Lehmann, K. P. Hunfeld, T. Emrich, G. Haberhausen, H. Wissing, A. Hoeft, and F. Stüber, "A multiplex real-time PCR assay for rapid detection and differentiation of 25 bacterial and fungal pathogens from whole blood samples," Med. Microbiol. Immunol. **197**(3), 313–324 (2008).
8. B. Suberviola, A. Marquez-Lopez, A. Castellanos-Ortega, C. Fernandez-Mazarrasa, M. Santibanez, and L. M. Martinez, "Microbiological Diagnosis of Sepsis: Polymerase Chain Reaction System Versus Blood Cultures," Am J Crit Care. **25**(1), 68–75 (2016).
9. P. A. Demirev, Y. P. Ho, V. Ryzhov, and C. Fenselau, "Microorganism identification by mass spectrometry and protein database searches," Anal. Chem. **71**(14), 2732–2738 (1999).
10. Atul Kothari, Margie Morgan, and David Haake, "Emerging technologies for rapid identification of bloodstream pathogens," Clinical Infectious Diseases (2014): ciu292.
11. T. H. Huang, X. Ning, X. Wang, N. Murthy, Y. L. Tzeng, and R. M. Dickson, "Rapid cytometric antibiotic susceptibility testing utilizing adaptive multidimensional statistical metrics," Anal. Chem. **87**(3), 1941–1949 (2015).

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12. M. Cartwright, M. Rottman, N. I. Shapiro, B. Seiler, P. Lombardo, N. Gamini, J. Tomolonis, A. L. Watters, A. Waterhouse, D. Leslie, D. Bolgen, A. Graveline, J. H. Kang, T. Didar, N. Dimitrakakis, D. Cartwright, M. Super, and D. E. Ingber, "A Broad-Spectrum Infection Diagnostic that Detects Pathogen-Associated Molecular Patterns (PAMPs) in Whole Blood," EBioMedicine. **9**, 217–227 (2016).
13. C. C. Neacsu, J. Dreyer, N. Behr, and M. B Raschke,, "Scanning-probe Raman spectroscopy with single-molecule sensitivity," Phys. Rev. B **73**(19), 193406 (2006).
14. W. Ho, "Single-molecule chemistry," J. Chem. Phys. **117**(24), 11033–11061 (2002).
15. T. Liu, Y. Lu, V. Gau, J. C. Liao, and P. K. Wong, "Rapid antimicrobial susceptibility testing with electrokinetics enhanced biosensors for diagnosis of acute bacterial infections," Ann. Biomed. Eng. **42**(11), 2314–2321 (2014).
16. J. T. Winskas, H. Wang, A. Zhdanov, S. Cheemalapati, A. Deonarine, S. Westerheide, and A. Pyayt, "Different regimes of opto-fluidics for biological manipulation," Micromachines **10**(12), 802 (2019).
17. L. D'Amico, N. J. Ajami, J. A. Adachi, P. R. C. Gascoyne, and J. F. Petrosino, "Isolation and concentration of bacteria from blood using microfluidic membraneless dialysis and dielectrophoresis," Lab Chip **17**(7), 1340–1348 (2017).
18. M. A. Faridi, H. Ramachandraiah, I. Banerjee, S. Ardabili, S. Zelenin, and A. Russom, "Elasto-inertial microfluidics for bacteria separation from whole blood for sepsis diagnostics," J. Nanobiotechnol. **15**(1), 3 (2017).